

The University of Chicago

THE INDICATOR METHOD OF MEAS-
URING OXIDATION-REDUCTION
POTENTIALS IN ALGAE

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BOTANY

1931

By
HENRIETTA LOUISE ZOBEL

Private Edition, Distributed by
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INTRODUCTION

Life has been thought of as an oxidation since the latter part of the eighteenth century when it was proclaimed that the one characteristic of living organisms is their capacity to oxidize substances incapable of oxidation at ordinary temperatures. It was soon shown, however, that all oxidations do not involve the element oxygen, and in living cells especially was this found true. A great many are of the electrolytic type where merely an exchange of electrons occurs. Because of this double meaning of the term Cady and Taft (3) have suggested that the term "oxidation" be restricted to those processes only in which oxygen is involved, while for the electrolytic type the terms "electronation" and "de-electronation" be used to designate reduction and oxidation respectively where an exchange of electrons occurs. However, in dealing with the oxidation processes of organic substances, and chiefly with those manifested by plant cells one finds but few of them to be strictly of the electrolytic type, and for these materials the older and broader terms seem more appropriate.

Most of the work in actual measurement of oxidation-reduction potentials has been done in the inorganic field, but recently there is shown a marked increase in the amount of work done with organic material. Life is being explained more and more as a mere physical interaction of the proper

materials, and the question of electron exchange rightfully deserves an important place.

For accurate measurements of electron exchange an insertion of the electrodes into the material is necessary, but since this method often involves certain difficulties when one deals with living material, the colorimetric method offers an easy shortcut with a degree of accuracy fairly comparable to the electrometric method. Certain dyes have long been known to change color with the environment in which they find themselves, but only in very recent years have the causes of such changes been determined and the rates of change been measured. Methylene blue especially has maintained a foremost position from the time it was first used as a vital stain up to the present, but rather fortunately its popularity is now declining and other more stable and more dependable indicators are coming into use. Theoretically these indicators themselves are oxidants or reductants, each with its own ease or difficulty of electron exchange. The color of the dye depends upon the degree of oxidation. The number of such dyes available for use as indicators is now fairly large, and new indicators are constantly being revealed. The difficulties in the use of some of these are still rather great, but biologists must rely upon such as are now available in the hope that our knowledge about them and their use will soon increase. This article is intended to mention some of the possibilities and complexities of the problem rather than

to state a series of indisputable facts. Some of the methods available to investigators in biology are described, and a few of the results attained by their use on certain plant cells are given.

In reviewing the literature one finds a large number of contradictory results. Some of these contradictions may be due to error, but it seems possible that all may be correct under the circumstances. When one measures the potential of a living cell one measures those processes which are strictly reversible only. Since having been conclusively shown that some processes are of this type it was so easy to hope that all would be; and we find numerous attempts to measure the potentials of cells by means of a method not applicable to the process. The results then must be conflicting. Even the result one does obtain cannot always be interpreted at present. How much of it is a result of injury and how much a result of the withdrawn oxygen (if done in an atmosphere of nitrogen) and how much is due to the stimulating effect of the dye on the protoplasm, seeing as it itself is capable of acting as well as of being acted upon, one cannot definitely say. The best one can do at present, it seems, is to perfect the method, and to interpret the results as best one can in the light of present information.

Very little work on the measurement of potentials has been done with plants. Somewhat more has been done with animal tissues. Needham and Needham (18) found the interior

of the cells of amoeba to have a pH of about 7.6 and an rH of between 17 and 19. Cohn, Chambers, and Reznikoff (6) injected twenty-five indicators into amoeba. They found that during anaerobiosis the amoebae were able to reduce all of the more easily-reduced indicators down to and including indigo disulphonate, but that not all of these could be re-oxidized. During aerobic conditions the rH may be entirely different from anaerobic conditions, as was found to be the case in one of the protozoon parasites of the frog. During anaerobic conditions the rH was found to vary between 9.5 and 10.5, while during aerobic conditions an rH between 19 and 30 was shown.

Brooks (2) found that some dyes can enter a cell only in the reduced form. The oxidation-reduction potential of the cell sap of Valonia was estimated to be between rH 16 and 18. Joyet-Lavergne (10) reported that the chondriosome is the center of the oxidation-reduction phenomenon of the cells of petals and of the pollen grains in *Antirrhinum* and *Capucine*. The chondriosome apparently is a carrier of glutathione, and has both oxidative and reductive powers; but Narutowicz (17) working with Fungi disbelieved the existence of oxidizing centers. Tamiya (20) in growing *Aspergillus* both aerobically and anaerobically found reductase present under both conditions, but always less so in the anaerobic. Glutathione measurements were also made. Joyet-Lavergne (12) also attributed respiration rates to

to glutathione. Those regions rich in glutathione are those in which carbohydrate metabolism occurs most rapidly.

Lutz (16) showed that tannins act as negative catalysts for oxidation, and he considered the presence of tannins in cells as important in helping regulate intracellular oxidations. Tannin centers are low in oxidizing rates.

Rapkins and Wurmsier (20) found no difference either in pH or in rH between the nucleus and cytoplasm when tested by injection of indicators. Other substances, such as fructose and glucose were also injected into cells while being watched for color changes, and no immediate change was found. apparently the rate of dehydrogenation in a cell is largely independent of the amount of hydrogen-donators in the cell.

Fildes (8) found that the spores of *B. tetani* do not germinate in a medium until this medium has reached a certain reductive intensity. Any point more positive than the required potential prevents germination. This is taken as the reason why spores of *B. tetani* do not germinate on subcutaneous fluids of guinea pigs, in so far as this is shown more positive than the germination point. Fuchs (9) attributed the reduction of methylene blue by yeast cells to the reductase contained. Reduction occurs more rapidly in an acid medium than in a neutral one. However he found methylene blue a poor indicator to identify the dead from the living cells, as even those cells which became colored (had no reducing power) produced buds under certain conditions.

Lund (13,14,15) worked on the relation of oxygen concentration to the oxidation-reduction ratio, and its effect on cell polarity. He finds that for frog skin the oxygen can be withheld temporarily without this altering the ratio, yet if anaerobic conditions are given for long periods the oxidation-reduction potential changes. Therefore the magnitude of electrical polarity may be increased or decreased by changing the concentration of the dissolved oxygen in solution. Lund also showed that in *Obelia* stem the rate of oxygen consumption and of carbon-dioxide emission is greater in the apical regions than in the basal portions. The apical part is thus electropositive while the basal region is negative. The potential difference in any cell is largely determined by the relative concentrations of oxidants and reductants, and the sum-total of all the potential differences of all the cells of the region (or tissue) determines its polarity.

Barron and Hoffman (1) showed that the oxidation-reduction indicators can be used as catalysts in living cells to increase the oxidative processes. This was measured by the rate of oxygen consumption, and if the cell walls are easily permeable and the indicator used is more positive than the potential of the cell under aerobic conditions a great catalytic effect can be shown.

Joyet-Lavergne (11) placed spores of *Equisetum arvense* into indicator dyes and finds them of two kinds. The

majority are very feebly colorable, indicating that they possess a lower potential than those spores which color deeply. On performing germination tests it was shown that female gametophytes were more numerous than male gametophytes, and therefore probably the difference in sex could be foretold in the reaction of the spores to dyes. Joyet-Lavergne also believes that there exists a sexualization of protoplasm, detectable by rH measurements. The female cells store reserves of fats, which have reducing ability. The male cells do not have this quality.

METHODS

The indicator method for measuring cellular rH seems to offer certain advantages. In the first place one can deal with much smaller quantities of material - a single cell. It also appears to be possible to insert the droplet of indicator into a special portion of the cell, e.g. the nucleus, the vacuole, or even to determine whether centers of oxidation exist about the chondriosomes. Especially did this become apparent when recently a large number of the oxidation-reduction reactions of plant cells were found to be strictly reversible. If all of them were strictly reversible then the possibilities for the indicator method would be even greater, but that is however not the case, and serious objections can be raised for depending entirely for information on a method which measures only a small portion of all the processes of that nature which occur in the plant.

In the selection of indicators one of the first requirements is that they be of low toxicity. Altho the color reaction is to occur in a fairly short time, even this small interval might materially alter normal life conditions. All evidence also seems to point to the fact that injury incurred in the prick of injection alters the equilibrium of the cell, but in spite of this, some fairly constant results seem to have been attained. In a search for indicators for

this work seven samples were graciously supplied by Dr. Mansfield Clark from his own stock for which the writer is now deeply indebted and thankful. And also the popular methylene blue was included. The eight used were as follows:

1. Indigo disulphonate
2. Indigo tetrasulphonate
3. Methylene blue
4. Toluylene blue
5. 1 Naphthol-2-sulphonate indo 3,5 dichlorophenol
(1 Naphthol-2-sulphonicacid indo-3',5'-dichlorophenol)
6. 1 Naphthol-2-sulphonate indophenol
(1 Naphthol-2-sulphonic acid indophenol)
7. 2,6,dichloro benzenone indo 3' methylphenol
(2,6, dichlorophenolindo-o-cresol)
8. 2,6 dichlorobenzenoneindophenol
(2,6-dichlorophenolindophenol)

Descriptions of the characteristics of each of these dyes can be found in the work of Clark and others (4).

Preparation of the indicator standards. Just as in the testing of H-ion concentrations by means of indicators one must have accurate color standards for comparison, so with oxidation-reduction indicators. Here, however, the matter is not so simple, as it is not merely necessary to have the

complete series of colors over the entire range from complete reduction to complete oxidation, but one must have such a series for each degree of H-ion concentration since the colors vary as much with pH as with reduction. The matter becomes more complicated yet thru the fact that these indicators do not change their color as from red to yellow, but merely vary in intensity of the same color. Much is left to the good judgement of the analyst even tho the concentration of his dyes is perfectly controlled and they are freshly prepared. Several methods for bringing about complete reduction were tried out as well as of preparing the color standards.

The double-wedge standard. This method was suggested by the double-wedge comparator for H-ions. The necessary wedges were obtained from a pH set in which the colors were believed no longer true to standard. The ends of these were broken and the liquids drained. These were thoroughly cleaned, and washed repeatedly with distilled water till no trace of the original acid or alkali remained. Then into one of the wedges was placed the completely oxidized form of the dye, dissolved in buffer solution, and the opening sealed with sealing wax. Into the other wedge was placed the completely reduced dye. Since the completely reduced form is clear only in a few dyes and is yellowish in most, the necessity of the presence of the reduced form in one compartment is

evident. To bring about complete reduction the easiest method was found to be by the use of titanous chloride. Glucose was also found extremely easy to manipulate.

A few indicators can be completely reduced in the presence of air, e.g. methylene blue. Others, such as acid indophenol are reduced with great difficulty, while indigo retains the oxidized appearance as long as it is in contact with any oxygen, even tho an excess of titanium be added. It becomes necessary, therefore to carry on reduction in an atmosphere free from oxygen. Nitrogen gas is preferred. Any closed chamber suffices for the reduction, but to avoid contact with air in transfer of the solution to the wedge it was found best to carry on the reduction directly in the wedge. It was placed into a vessel slightly larger than itself, one which could be closed securely except for the tubes leading to the nitrogen and the outlet tube. Buffer solution was used in the wedge with sufficient alkali to neutralize the acidity of the titanium to be added. Sufficient titanium to bring about complete reduction was added and then the nitrogen was allowed to bubble slowly thru the entire system until complete reduction was attained. On reduction the wedge was quickly sealed, avoiding any aggitation whatever. This made a nice color standard for it could be assumed that the central point would represent fifty percent reduction, and all others proportional.

The one great objection to this type of color standard

is that there are so few places in biological work where it can be used to advantage. Since it makes a great difference whether one is sighting thru one-half inch, two inches, or ten microns of solution, this standard is usable only for tubes having exactly the same diameter as the wedges. Test tubes of such size are available and for reduction studies carried on with bacteria grown in such tubes this works nicely, and is preferable to any other method in that it requires only the preparation of the completely reduced and the completely oxidized forms without the trouble of preparing each of the percentages separately. It is merely necessary to know the exact pH, and to make sure that the same concentration of dye is used in the wedge as in the tube. However, if titanium is used the wedges do not keep well; and for permanency some other reducing agent is to be preferred.

The test tube standard. This series of standards has one advantage over the double-wedge method described above. In the former it is obvious that the fifty percent reduction point is in the center, but all other points are not proportional. Titration curves show that there is a stabilizing action going on near the fifty percent point, and that the greatest degree of change occurs near the one and ninety-nine percent regions. The scale on the wedge must therefore be calibrated into uneven distances. The test tube method removes much of this difficulty. The buffer solution and the oxidized form

of the dye were prepared as necessary. The reduced form was prepared according to the method and with the apparatus described by Needham and Needham (18). This assures complete reduction without any excess titanium being present. Success was also had when the zinc method of reduction was used.

Following reduction a layer of paraffin oil was poured over the dye which avoided further contact with air. As much could be drawn up into a pipette as needed without contact with oxygen as long as the oil layer preceeded. To prepare, say the seventy percent reduced dye, thirty portions of the oxidized form were placed into a test tube and seventy portions of the reduced form were added; and since the oil always protected the liquids it was possible to fill up the tubes to the top with oil and a tight cork could be added for further protection. If titanium was used, these standard tubes are no more permanent than the wedges, but if zinc was the reducing agent a somewhat more permanent form was attained. However, even this had a tendency to change in time, and for accurate work fresh solutions should be used.

The micro-pipette standards. This is similar in procedure to the test tube method up to the point of filling the final tubes. For microinjection work one requires a color standard of as nearly the same diameter of the cell under investigation as possible. It would be useless to try to compare the shade of color as seen in a cell ten microns in diameter when mag-

nified by a microscope. with a series of tubes one-half inch in diameter. Magnification also makes the color so diffuse that a tube small enough to allow similar magnification becomes necessary. The experimental error which creeps in during the use of such small quantities is so great that it tends to invalidate the apparent results. Also, the concentration of the dye used must of necessity be great. Adsorption comes in to play so easily that misinterpretations are almost inevitable.

Common sealing media such as melted wax or collodion adhered poorly to the ends of these tubes, and repeated attempts were made before being certain that the ends were actually sealed.

Preparation of the tubes themselves is no trick. A large number of such Pyrex glass tubes were drawn and those of proper diameter selected. Pieces a few centimeters in length are sufficient. By placing these pipettes on the slide beside the material studied one can compare the shade of color in the cell with that in these tubes.

Reducing agents. Not all the reducing agents were reviewed in this work, but a few comments can be given on the following:

1. Titanous chloride. Titanium is very commonly used as a mordant in the dye industry, as it is one of the most powerful reducing agents known. It is however an extremely unstable substance, and has a way of deteriorating almost

before the experiment is completed. It must be prepared fresh for every reduction. Especially is this true when mixed with buffer solution. The buffer is indispensable, and yet in alkaline solutions titanium hydroxide is formed which precipitates in purplish flocculations which do not help to produce clear colors.

An ingenious device for the use of titanium in reduction was found described by Needham and Needham (18), and which makes possible the use of all necessary reagents in an atmosphere of nitrogen and at the same time the use of the gas pressure from the nitrogen tank to direct the flow of each reagent. The principle behind this arrangement was copied for some of the reductions for this work.

2. Powdered zinc. Obviously, an indicator reduced with titanium cannot be used for injection purposes. Powdered zinc renders a reduced dye in very short time, and one which is usually reversible. The writer however finds that not all that appears to be reduction actually is reduction when zinc is used. Especially was this true for methylene blue, and to a lesser degree for all others. Methylene blue has a great affinity for glass and metals, and altho in the presence of powdered zinc all traces of color disappeared, on investigation one would find that the zinc particles had merely adsorbed the dye, and only a small portion of the original color could be reobtained by oxidation. The more concentrated

the dye, the more pronounced was this phenomenon of adsorption. In extremely dilute solutions it was negligible, but such dilute solutions are valueless for injection purposes. Consequently it becomes impracticable to mix methods, e.g. using titanium for reduction in the making of the color standards and using zinc for reduction of that indicator to be used for injection, for tho one may start with solutions of the same concentration the chances are that they will be different on finishing.

Reduction with zinc can be induced with very simple equipment. A test tube and water bath suffice. The dye, dissolved in buffer solution was placed into the tube, and a small quantity of powdered zinc added. This was placed into a vessel so equipped that a slow stream of nitrogen gas could bubble through for a while. When the dissolved oxygen was reduced to zero the tube was quickly covered with oil and placed into a water bath. Reduction occurred rather easily, and began at a temperature considerably below boiling.

3. Aluminum and mercury. Aluminum filings or aluminum wires were boiled with dilute sodium hydroxide, washed, and covered with water. When ready for use the filings were placed in mercuric chloride solution and heated to the boiling point for a few minutes. The liquid was decanted off, and the mercury-covered filings washed and kept covered with water. For reduction they were placed into the dye, and a

rapid evolution of gas took place during which the dye was reduced. As this soon decomposes it was necessary to withdraw the liquid from the top as soon as reduction was complete. Reduction brought about by this method yields a dye not suitable for use in microinjection due to the toxicity of mercury, but in preparing color standards it proved easy and was reversible.

4. Glucose. Efimov (7) used glucose in the determination of the oxygen content of different solutions. This suggested the use of glucose as a reducing substance for indicators. But, as it seemed that the use of glucose solutions would have but few possibilities for use in microinjection studies, only the general methods were worked out. It has also been found that levulose would reduce certain indicators, but only those within a certain range of potential.

One gram of glucose was dissolved in a one percent solution of potassium carbonate. The indicator was dissolved in this solution and within one hour reduction was well on the way in the more easily reduced dyes. Within twenty-four hours reduction was complete in all of the dyes used.

5. Silver chloride. One of the suggested contact catalysts is silver chloride, which, it is claimed causes an immediate reduction with an evolution of chlorine. This substance was, however not tried out.

6. Oxides of platinum or of palladium, obtained by treating chloroplatinic acid crystals with sodium nitrate forms a precipitate which has catalytic properties in reduction. This was not repeated.

Microinjection needles. The whole trick in preparing injection needles seems to lie in not drawing the needle at one heating, for that gives a needle perhaps sharp enough but the point is so long as to be useless. A needle can be made in which the point is no longer than ten millimeters from the place where it begins to taper. Such needles are sharp, stiff, and liquid can be forced thru such an orifice, while if longer, the capillary attraction is so great that the squeezing out of a droplet of liquid is practically impossible.

The method by which needles were prepared is as follows: Pyrex glass tubing was held over a gas microburner, the opening of which was the size of a small pinhole. The flame was adjusted so that it was never more than one-fourth inch in height. The glass rod was held in the flame till slight drawing was possible. Then the rod was withdrawn and allowed to cool. The second heating took place at a point to one side where the tapering was just beginning, and again it was drawn but a slight distance and again allowed to cool. One end of the rod will thus become a rather long needle which is useless, while the other yields one that tapers abruptly.

The last drawing is the most important, and the slightest degree of heat only in the most localized of places is necessary. The needle is then broken at the sharpest point. The bending process is quite simple. It merely means that the point must be protected from the flame. A small piece of metal is inserted under the point as it is placed over the microburner, and slight pressure against this metal will turn the point slowly upward until the desired bend is attained. A neat right-angled bend can thus be made in the wider portion of the tube.

The use of a moist chamber is desirable for two reasons: it acts as a trap for the nitrogen gas when such is used, and also keeps moist the material studied. Such moist chambers can be obtained, or can be constructed with very little effort out of a few pieces of cut glass sealed together properly.

One micromanipulator was found sufficient. The injection needle was inserted into rubber tubing, and this attached to a metal tube on the end of which was a rubber bulb. When the needle was attached to the manipulator the bulb was placed aside and this could receive the proper amount of pressure without dislogging the remainder of the set-up. When injections were made in the presence of nitrogen a small tube led into the moist chamber and a slow stream of gas thus flowed about the tissue.

This method is subject to great criticism, and the author feels that many improvements are necessary. Also, at present very little light is shed on the life processes of cells thru measurement of the cell's rH, and probably only a very limited number of the cell's activities can be measured by this method. Not all processes are strictly reversible. Therefore the indicator method is one entirely inappropriate to use until we know exactly which particular reaction one is determining by this method. That so many investigators working on different species should have obtained similar results may be looked at as a result of accuracy, but, it might also be taken to signify that reactions are fundamentally alike in all cells, and individual peculiarities are not touched at all by these measurements.

That distinctly different results are obtainable when the cell is aerobically grown from those when anaerobically grown indicates that at least some change is occurring, and an atmosphere of nitrogen cannot be taken as a normal environment for aerial plants. Indicators which can be used in the presence of oxygen would do away with this difficulty. The majority of plant cells have some free oxygen content. That this free oxygen has a respiratory function can hardly be disputed, even the respiration may temporarily be induced to occur anaerobically. But indicators can be used only after the free oxygen is removed from the cell. The author feels that the oxygen is as much a part of the cell

as is the protoplasm, and should be taken account of in respiration measurements.

The indicators themselves, being active substances, may become catalysts, and therefore not all results are the result of the action of cellular material on indicators, but the result of indicators on cellular material.

Adsorption plays so large a part that the experimental error becomes enormous. Also, judgement of colors is at best but guesswork, and anyone trying to determine shades of color in a cell filled with chloroplasts must possess excellent powers of discrimination. The personal element becomes large, and error creeps in easily.

Injury is pronounced. Puncturing a cell is necessary, and acute disturbances are inevitable.

In spite of these difficulties the writer still feels that the indicator method is one offering great possibilities. With the hope that in the future many of these difficulties be removed or interpreted, the writer undertook to use this method at its present state of perfection in investigation of certain cell interiors of Algae.

Algae were chosen for microinjection studies primarily for three reasons: 1. They are unicellular, or filamentous, and it is possible to deal with material consisting of isolated cells without the necessity of cutting and thereby perhaps changing the entire equilibrium of the region of study. 2. The cells are large, especially the cells of the

algae selected: *Vaucheria*, *Hydrodictyon*, and *Gladophora*.

3. It might be possible to find differences in sexuality.

In *Vaucheria* the antheridia and oogonia are large, and are on the same filament. If sex is a very local phenomenon as it appears to be in such a monoecious plant then perhaps the relation between sex and rate of metabolism could be discovered. In *Spirogyra* this possibility is also apparent. Tho there is no distinctly dioecious condition in *Spirogyra*, yet during conjugation one filament acts as tho it were "male" and the other "female". Perhaps there are two distinctly different filaments which could be identified by their potential. Such differences in rates of metabolism between males and females are quite conspicuous in higher animals, but little is known of this phenomenon in plants.

The pH of the cell interior was determined by measuring it in several of the cells of the species, and assuming that those cells used to inject oxidation-reduction indicators possess the same pH. Color is very difficult to judge. Algae are rich in plastids and there is no place in the cell where these are not present in large numbers. As indicator such as brom-thymol blue was soon given up as too inaccurate. Comparisons were made with another uninjected cell by the side of the injected one to compare the shades of green. Somewhat better results were obtained with neutral red. Placing the indicator before a chlorophyll solution was also abandoned as it seemed to give no greater degree of accuracy.

The plastids themselves remained as islands of clear green, and there was no such uniform admixture of colors.

For microinjection the strand of alga was placed on a large coverglass which fit over the top of the moist chamber. The filament was brought into focus, and the pipette adjusted in its holder so that the point of the needle was just under the cell in question. Only slight turning of the screw raised the needle and forced its point into the cell. To prevent the slipping of the filament which occurred as the needle touched it, two square coverglasses were attached to the large coverglass by means of a thin film of vaseline. Between them was placed the filament, and the two coverglasses could be pushed slowly so as to line up snugly on each side of the filament. This held the strand securely in place while the needle entered.

That considerable injury occurred was apparent, for following the injection of a small quantity of indicator and withdrawal of the needle there could usually be seen the oozing out of some of the cell contents at the place of puncture.

Readings varied little between the three algae studied, and were not always uniform among themselves. All were distinctly alkaline, varying between pH 7.4 to 8.0. pH 7.6 and 7.8 were taken as the average around which all cells fluctuated. Readings of cellular pH were also made by placing the filaments entirely into the indicator and allowing

this to be absorbed for about thirty minutes. Some produced good coloration, but it seemed as tho most of the color was in the cell wall itself. Readings were always slightly higher this way than by injection.

A buffer solution adjusted to pH 7.8 was prepared of Na_2HPO_4 , HCl , and H_2O . Buffer and reducing solutions were prepared fresh, as they do not keep well.

When pipettes were first filled with the various percentages of reduced dyes, all those more than 50% reduced appeared white. One is dealing with quantities so small that colors must be dark indeed before they are even visible in such minute tubes. Obviously the concentration of the dyes must be increased, and increased almost to a dangerous degree. The exact concentration of the dyes was not noted since it really didn't matter. The author believes this increased concentration subject to great criticism because it increases the experimental error, it may be more toxic to the cells, and it increases the chances of adsorption. It also requires longer time for reduction, and when approaching the higher degrees of reduction this also is subject to the same errors in interpretation of color intensity.

Results were not always uniform, but the average was taken based on a fairly large number of injections. The oxidized form of the dye is of course much easier to work with, and most of the injections were made with this form. There was always a quick appearance of color when the reduced form

was injected into the cell and nearly always this was quite identical with the color produced on the injection of the oxidized form. It always seemed to take longer for them to become reduced than to become oxidized - oxidation occurring almost instantly, but reduction not being complete until after fully one minute. Injections were always carried on in an atmosphere of nitrogen, even tho the use of nitrogen is one of the most serious objections in the use of such indicators. Those life processes depending upon the presence of free oxygen must be either discontinued or altered. Perhaps we shall some day have available indicators which act independently of air, involving merely a transport of electrons.

RESULTS

Injection of Cladophora. A large species of Cladophora was used. None of the cells of this material were producing gametes or spores, so only the vegetative condition could be studied. Filaments were mounted according to the method described, and nitrogen was allowed to flow in a very slow stream around the filament for about half an hour before injection was begun.

At pH 7.8 the indicators should become reduced in the following order, and the dyes were used in this order:

1. 2,6 dichlorobenzeneindophenol
2. 2,6 dichlorobenzeneindo 3' methylphenol
3. Toluylene blue
4. 1-naphthanone-2-sulphonic acid indophenol
5. 1-naphthanone-2-sulphonic acid indo 3',5' dichlorophenol
6. Methylene blue
7. Indigo tetrasulphonate
8. Indigo disulphonate

These will be referred to by number only in the discussion following.

No. 1 was apparently completely reduced. In two cases this was distinctly not the case, but even here reduction seemed to have occurred to about 90 percent.

No. 2 was also completely reduced in nearly all the

cells. Three hovered around the 90% point.

No. 3 was completely reduced in some; in others only about 90%, while in one it retained color enough to signify about 80% reduction.

No. 4 gave readings similar results to No. 3. They varied from 80% reduction to complete reduction in one instance.

No. 5 showed about 80% reduction in the majority of cells. Others varied between 70% to 90%.

No. 7 showed no reduction.

No. 8 showed no reduction.

TABLE I
REDUCTION OF INDICATORS BY CLADOPHORA CELLS

Indicator	Average reduction	Average oxidation	E_h at 50% red.	Correction	Corrected E_h
1	99%	1%	0.165	-0.0902	.0748
2	99%	1%	0.125	- .0600	.0650
3	95%	5%	0.088	- .0492	.0388
4	90%	10%	0.074	- .0287	.0453
5	80%	20%	0.060	- .0181	.0419
6	5%	95%	-0.014	+ .0384	.0244
7	0%	100%	-0.160		
8	0%	100%	-0.077		
Average E_h 0.048366					

Injection of Hydrodictyon. The pH of Hydrodictyon was found to vary between 7.5 and 7.8. The average of 7.6 was used in the preparation of the buffer.

The same general plan was followed in these injection studies as for Cladophora. It was found necessary to select only the larger cells, but at this time of year none of them were forming zoospores; hence the figures are for vegetative stages only. The order of reduction of dyes at this pH is the same as for 7.8 listed above. Average reductions are given in Table II.

TABLE II
REDUCTION OF INDICATORS BY HYDRODICTYON CELLS

Indicator	Average reduction	Average oxidation	E_h at 50% red.	Correction	Corrected E_h
1	95%	5%	0.175	-.0384	.1366
2	85%	15%	0.138	-.0234	.1146
3	10%	90%	0.094	.0287	.1227
4	10%	90%	0.087	.0287	.1157
5	2%	98%	0.073	.0550	.1280
6	0%	100%	-0.008		
7	0%	100%	-0.070		
8	0%	100%	-0.152		
Average E_h 0.12352					

Injection of Vaucheria. Vaucheria was selected primarily to make possible the study of antheridia and oogonia. The pH was found to be about 7.8. The indicators were prepared in buffer solution as previously described, and all injections were made in the same way. Three areas were investigated: The vegetative cells, the antheridia, and the oogonia. Results for Vaucheria are tabulated in Table III, IV, and V.

TABLE III

REDUCTION OF INDICATORS BY VAUCHERIA VEGETATIVE CELLS

Indicator	Average reduction	Average oxidation	E_h at 50% red.	Correction	Corrected E_h
1	100%	0%	0.162		
2	95%	5%	0.125	-.0384	.0866
3	95%	5%	0.088	-.0384	.0496
4	90%	10%	0.074	-.0287	.0453
5	80%	20%	0.060	-.0181	.0419
6	1%	99%	-0.014	.0600	.0460
7	0%	100%	-0.090		
8	0%	100%	-0.174		
Average E_h 0.05388					

TABLE IV

REDUCTION OF INDICATORS BY VAUCHERIA ANTHERIDIA

Indicator	Average reduction	Average oxidation	E_h at 50% red.	Correction	Corrected E_h
1	100%	0%	0.162		
2	99%	1%	0.125	-.0600	.0650
3	98%	2%	0.088	-.0546	.0334
4	90%	10%	0.074	-.0287	.0453
5	85%	15%	0.060	-.0234	.0366
6	3%	97%	-0.014	.0492	.0352
7	0%	100%	-0.090		
8	0%	100%	-0.174		
			Average E_h 0.0431		

TABLE V

REDUCTION OF INDICATORS BY VAUCHERIA OOGONIA

Indicator	Average reduction	Average oxidation	E_h at 50% red.	Correction	Corrected E_h
1	99%	1%	0.162	-0.0600	.1020
2	97%	3%	0.125	-0.0492	.0758
3	95%	5%	0.088	-0.0384	.0496
4	95%	5%	0.074	-0.0384	.0356
5	80%	20%	0.060	-0.0181	.0419
6	1%	99%	-0.014	0.0600	.0460
7	0%	100%	-0.090		
8	0%	100%	-0.174		
			Average E_h 0.05848		

Determination of rH. rH can be defined as the "negative logarithm of the hypothetical hydrogen pressure in equilibrium with the oxidation-reduction system in question at a given pH". Or

$$rH = \log \frac{1}{P}$$

As has already been shown, the potential varies with the pH, and altho it is not an entirely uniform fluctuation, with each unit increase in pH the potential becomes about 0.06 more negative.

Clark (4) has devised a diagram on which the relation between E_h , pH, and rH can be shown at once. By means of such a diagram one can readily convert any E_h figure at any pH into the corresponding rH. Thus, converting all the E_h readings obtained for the algae, one obtains the following as average readings:

TABLE VI
rH OF ALGAL CELLS

	pH	E_h	rH
Gladophora	7.8	0.04836	15
Hydrodictyon	7.6	.12352	12
Vaucheria vegetative	7.8	.05388	14
Vaucheria antheridium	7.8	.04310	15
Vaucheria oogonium	7.8	.05848	14

Effect of indicators on germination. The work of Rapkine (19) which consisted of substituting methylene blue for the oxygen naturally contained in sea water was suggestive. Rapkine placed eggs of Oursine in boiled water, and found no further development could take place under these anaerobic conditions, but when methylene blue was added to the boiled water before being sealed from further contact with oxygen normal development took place in the eggs.

That seeds will not germinate normally in water boiled to free it of oxygen is a long established fact. Methylene blue is known as a hydrogen-acceptor. If this is true then if methylene blue be added to seeds during their germination it might be possible not only to have respiration initiated on an anaerobic basis, but perhaps to continue for some time without access to free oxygen provided the rate of oxidation could be altered by some catalyst. Thus, the corresponding incident in the plant kingdom might be accomplished which seems to have been done in the animal kingdom with the eggs of sea animals.

Experiments were carried out on the germination of wheat under anaerobic conditions, and the influence of indicators in their oxidized form upon germination. Theoretically, according to Rapkine, as the indicators are reduced by the life processes the cell oxidations would occur, and by the use of indicators of known potential the potential of the germinating seed might thus become established and at the same time

be measured.

Germination of seeds occurred under strictly sterile conditions. They were germinated in 100cc. Erlenmeyer flasks each plugged with cotton to prevent contaminations. Seeds were germinated under eleven conditions, eight of which had sufficient indicator added to impart a fairly deep color to the liquid.

Lot 1. In sterile water, aerated, and kept in the presence of oxygen. Aggitation was frequent.

Lot 2. In sterile water, boiled to remove oxygen, but not subsequently sealed from contact with air.

Lot 3. In sterile water free from oxygen, and sealed from further contact with oxygen.

Lot 4. Same as lot 3, but with 2,6,dichlorobenzenone-indophenol added.

Lot 5. Same as lot 3, but with 2,6, dichlorobenzenone-
indo 3'methylphenol added.

Lot 6. Same as lot 3, but with 1 naphthol-2-sulphon-
ate indophenol added.

Lot 7. Same as lot 3, but with 1 naphthol-2-sulphon-
ate indo 3,5, dichlorophenol added.

Lot 8. Same as lot 3, but with toluylene blue added.

Lot 9. Same as lot 3, but with methylene blue added.

Lot 10. Same as lot 3, but with indigo tetrasulphon-
ate added.

Lot 11. Same as lot 3, but with indigo disulphonate

added.

Contact with oxygen was prevented by pouring a layer of oil over the top of each, with the exception of the first two. All were kept at a temperature of about 20 - 25°C for five days. After 24 hours a very perceptable reduction had occurred in several of the dyes. After 48 hours pronounced adsorption was visible in lot 6 and in lot 8. That which contained methylene blue showed a more marked adsorption than any of the others, the seeds being more deeply pigmented than the original solution had appeared.

After four days germination had taken place only in those seeds which were growing in the repeatedly aerated flask, and even those germinated rather poorly. Most of the liquids were not decolored, yet all of them showed perceptable loss in intensity of color. Apparently, oxidizing catalysts such as these indicators, are insufficient to induce respiration on an anaerobic basis and make it continue so up to such a time when germination may be witnessed by the appearance of the embryos through the seed coats. It was not possible, therefore to reproduce in the plant kingdom with seeds what seems to have been done with animal eggs.

SUMMARY

1. The use of the indicator method for measuring oxidation-reduction potentials in plant tissues is reviewed and the advantages derived from its use are pointed out.

2. Three methods for the use of indicator standards are described: the double wedge method, the test tube method, and the micropipette method, with the advantages and disadvantages in the use of each pointed out.

3. The use of titanous chloride, powdered zinc, aluminum and mercury, glucose, silver chloride, and oxides of platinum or palladium as reducing agents is described with suggestions for the use of each.

4. The use of the microinjection needle for injection is described and a method for preparing such needles is given.

5. Three algae were used for microinjection studies: *Vaucheria*, *Hydrodictyon*, *Cladophora*. The pH of *Cladophora* and of *Vaucheria* was found to be about 7.8, while that of *Hydrodictyon* was found to be about 7.6.

6. The oxidation-reduction potentials found were: *Cladophora* rH = 15; *Hydrodictyon* rH = 12; *Vaucheria* antheridium rH = 15; *Vaucheria* oogonium rH = 14; *Vaucheria* vegetative rH = 14.

7. Indicators were found to have no stimulating effect on germination of the seeds of wheat.

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